AMENDMENTS TO THE SPECIFICATION:

Please delete the paragraphs (in their entirety) beginning at page 23, line 26, which starts with "Figure 1" through page 30 and replace with the following amended paragraphs:

Figures 1a-1d. E1A binds a 12 amino acid motif in CBP (amino acids 1811-1822). **Fig. 1a,** schematic representation of the transcription factor CBP showing an amino acid region (between 1621-1877) that binds the factors E1A, p53, E2F, and TFIIB. **Fig. 1b,** GST-CBP fusion constructs used in pull-down experiments to define sequences capable of binding 12S E1A. The bacterial GST-fusion proteins were bound to micro-columns and *in vitro* translated ^{35}S-labeled E1A passed over them to detect protein-protein interactions (see methods). Approximately 10% of the E1A translation reaction was run in the input lane, GST represents a control column, and lanes 1 to 9 represent the eluate obtained after passing E1A over columns containing the nine GST-CBP fusion constructs. **Fig. 1c,** Further deletion analysis of CBP sequences 1808-1826. Thick lines indicate constructs that bind E1A, narrow lines those that do not. A minimal construct of 12 amino acids (construct 10, CBP 1811-22) still retains E1A binding activity. **Fig. 1d,** Mutagenesis of the CBP TRAM. Indicated are the eleven alanine substitutions used to determine amino acids required for the E1A interaction. All are single substitutions, except for mutant construct 1, which substitutes two basic residues. Pull-down experiments using wild-type or mutant GST-CBP (1808-1826) sequences were carried out and show that E1A binding is abolished by the R1811A, K1812A mutation and the

N1814A mutation. Also shown is the amount of GST-CBP protein used in the pull-down experiment.

Figures 2a-2d. Identification of the amino acids in E1A involved in the interaction with CBP.

Fig. 2a, schematic representation of 13S E1A depicting conserved regions CR1, CR2, and CR3. Contained within CR1 are residues 63-67, previously implicated in the binding of CBP. GST-E1A proteins containing wild type or mutated (alanine substituted) sequences were tested for their ability to interact with ^{35}S-labeled CBP (1621-1877). Shown are the results of these pull-down experiments along with a picture of a coomassie-stained SDS PAGE gel to show the quantities of GST-E1A fusion proteins recovered from the micro-columns. Double substitution mutants within sequences F65 to L71 fail to bind CBP. **Fig. 2b,** Peptide competition assays confirm the involvement of sequences F65 to L71 in the interaction with CBP. Peptides of 30 amino acids containing the sequences shown above were used in competition studies to test their ability to prevent the E1A-CBP interaction. Increasing amounts (0.1mM, 0.5mM, and 1mM) of wild type peptide sequence (WT), or mutant sequences (Mut 1 or Mut 2) were used in E1A-CBP pull-down assays. Only the wild type peptide prevented the E1A-CBP interaction. **Fig. 2c,** p53, E2F, and TFIIB all bind to CBP sequences 1808-1826 that contain the TRAM. Binding can be inhibited by competition with a wild-type E1A peptide, but not by the Mut 2 E1A peptide. **Fig. 2d,** An alignment of E1A, p53, and E2F

sequences with the conserved FXE/DXXXL TRAM interaction motif (TRIM) underlined.

Figures 3a-3d. Mdm2 contains a C-terminal TRAM that binds E1A, p53, E2F, and TFIIB.

Fig. 3a, An alignment of sequences from human Mdm2 (466-484) and CBP (1808-1826). Conserved sequences are boxed and residues previously mutated (Figure 1d) to prevent E1A interaction are denoted by an asterisk. **Fig. 3b,** *In vitro* binding of E1A, p53, E2F, and TFIIB to the Mdm2 TRAM. Pull-down experiments show all four *in vitro* translated and radiolabeled proteins bind the wild type Mdm2 TRAM contained within sequences 466-484. A mutant GST-Mdm2 construct (N472A) fails to bind E1A, like its CBP counterpart (Figure 1d). **Fig. 3c,** The C-terminal Mdm2 TRAM is masked by N-terminal sequences between 391-421. Shown is a schematic representation of full-length Mdm2 containing the N- and C-terminal regions capable of binding p53, along with constructs used in *in vitro* pull-down experiments. GST-Mdm2 fusion constructs containing Mdm2 sequences 223-491 and 391-491 show vastly reduced binding capabilities compared to the unmasked C-terminal region 421-491. Also shown for comparison is the use of the N-terminal 1-125 amino acid region of Mdm2, previously shown to interact with both p53 and E2F. **Fig. 3d,** *In vivo* interaction between Mdm2 C-terminal sequences (421-491) containing an unmasked TRAM, and p53. MRC5.SV40 cells were transfected with 0.1 μg or 0.5 μg of either a pCMV-GST-Mdm2 (421-491)

construct, or a control CMV-GST construct. After 48 hours, cell lysates were prepared from these cells and incubated with glutathione-Sepharose beads, then subjected to washes and elution as described for *in vivo* pull-downs (see methods). Eluted proteins were run on SDS polyacrylamide gels and subjected to standard western blot analysis using the p53 monoclonal antibody DO-1. (Santa Cruz). Lysate containing transfected GST-Mdm2 (421-491) indicated complex formation between p53 and Mdm2 (421-491), while those from the GST control transfections failed to pull-down p53 protein. Lysate approximating to 10% of the amount loaded onto micro-columns was loaded directly onto the same SDS polyacrylamide gel and subjected to the same western blot analysis. A comparison between p53 levels detected suggests that approximately 2-3% of total cellular p53 is complexed with the GST-Mdm2 fusion protein.

Figures 4a-4d. The CBP and Mdm2 TRAMs compete for p53 binding with the N-terminal domain of full-length Mdm2 and activate p53-dependent transcription.
Fig. 4a, Differential effect of the p53 mutant 14/19 on the Mdm2 N-terminal domain and the CBP and Mdm2 TRAMs. *In vitro* translated ^{35}S-methionine labelled p53, either wild type or harbouring the L14Q/F19S mutation, were analysed for their ability to bind to GST-fusion proteins containing the N-terminal Mdm2 domain (1-125), the CBP TRAM (1715-1852), or the Mdm2 TRAM (421-491) in pull-down assays. While the binding of p53 to the N-terminal domain of Mdm2 is drastically affected by the 14/19 mutation, neither the CBP nor the Mdm2 TRAMs are affected. **Fig. 4b,** A CBP TRAM peptide can

inhibit the binding of the N-terminal Mdm2 domain to p53. CBP peptides of 27 amino acids (1806-1832) containing either wild type or mutant (R1811, K1812, N1814) TRAM sequences were used in competition assays to prevent the interaction of *in vitro* translated p53 and GST- Mdm2 (1-125). Wild type peptide was able to completely inhibit the p53-Mdm2 interaction over the range used (10-100 μM), while the ability of mutant TRAM peptide to inhibit the interaction was severely impaired. **Fig. 4c,** CBP sequences containing a wild type TRAM activate p53-dependent transcription. Transient transfection of U-2 OS cells was carried out using either 2 μg of a p53-responsive reporter gene (PG13CAT) or a control vector (MG15CAT). Also indicated is the co-transfection of 1, 2 or 4 μg of a CMV-GST-CBP (1808-1852) vector containing either a wild type TRAM, or a mutant TRAM (R1811A, K1812A). Introduction of the wild type TRAM resulted in a dose-dependent increase in p53-dependent transcription. This level of activation was not obtained when the mutant TRAM construct was used. The co-transfection of full-length Mdm2 abolished TRAM activation of p53 by CBP. **Fig. 4d,** U-2 OS transfection experiments demonstrate that introduction of unmasked Mdm2 TRAM sequences result in a similar activation of p53-dependent transcription. In addition to PG13CAT, co-transfections were carried out using 2 μg of the CMV-GST-Mdm2 expression vector (containing Mdm2 sequences 421-491, 391-491, 223-491, 1-491, or 1-125). It can be seen that transcription from the p53-dependent reporter construct is activated upon co-transfection of the Mdm2 421-491 construct that contains a functional TRAM. This effect is reduced upon the inclusion of N-terminal masking

sequences (see 391-491 and 223-491). Co-transfection of the N-terminal motif of Mdm2 alone also activated p53-dependent transcription, while full-length Mdm2, containing sequences that lead to p53 degradation, resulted in a reduction in p53-dependent reporter activity.

Figures 5a-5c. YY1 fragments containing sequences showing marked similarity to previously defined TRIMs are able to interact with CBP *in vitro* and are capable of repressing AP-1 activity *in vivo*.

Fig. 5a, Schematic representation of the YY1 constructs used in *in vitro* pull-down assays and in *in vivo* repression assays. WTYY1 represents the full-length wild type YY1 sequence, YY1BP, YY1BS, and YY1BH, start at amino acid position 1 and terminate at unique restriction sites within the YY1 sequence, while YY1ZF 2-4 and 1-4 represent PCR amplified YY1 sequences containing either all 4 zinc fingers (ZF 1-4) or the last 3 zinc fingers (2-4). These sequences were either fused to GST or GAL4 (1-147) sequences for the assays described below. **Fig. 5b,** In vitro pull-down assays demonstrate the ability of fragments containing at least one putative TRIM sequence to interact with IVT CBP (1621-1877), while the fragment YY1BP which does not contain a TRIM sequence, fails to interact with CBP. **Fig. 5c,** YY1 fragments capable of binding CBP *in vitro* demonstrate an ability to repress AP-1 activity *in vivo*. The schematic drawing shows the two palindromic GAL4 binding sites that were used to replace naturally occuring YY1 sites in the p80:2e/9e CAT reporter construct (O'Connor *et al.*, 1996).

This p80:2e/9eGAL reporter construct was then co-transfected with plasmids capable of expressing GAL-YY1 fusion proteins under the direction of an SV40 promoter in primary human keratinocytes. It can be seen that the expression of GAL-YY1 fusion constructs containing TRIMs results in a repression of CAT activity compared to those results obtained when only GAL sequences are co-transfected. By contrast the co-tranfection of a GAL-YY1BP construct which possess no TRIM fails to show any significant repression of CAT activity

Figures 6a and 6b. Differential binding properties of CBP TRAM mutant sequences to TRIM containing proteins.

Fig. 6a, A sequence alignment of previously defined TRIMs along with the putative TRIM sequences present in TFIIB, YY1 and MyoD. The phenylalanine residue and acidic residue in postions 1 and 3 respectively are highly conserved, while the leucine residue in the 7^{th} position sometimes shows a conservative change with another neutral, non-polar amino acid residue. Also illuminated is the large variation in amino acid residue composition in the remaining TRIM sequences, and in the surrounding sequences. **Fig. 6b,** *In vitro* pull-down assays demonstrate the failure of TRAM C-terminal residue alanine substitutions to bind a subset of TRIM-containing proteins.

Figures 7a-7d. HPV-16 E6 interacts with the transcriptional co-activator CBP/p300. (**Fig. 7a**) Equal amounts of partially purified full-length (FL) CBP/p300 from HeLa

nuclear extract was passed over GST, GST-16E6, GST-P/CAF, and GST-YY1 micro-affinity columns (see Materials and Methods). After SDS-gel electrophoresis and transfer to membranes, western analysis detected the presence of CBP/p300. (**Fig. 7b**) GST micro-affinity columns were used to detect the interaction of in vitro translated and radiolabeled HPV-16 E6 with GST-CBP II (residues 1621 to 1877). No interaction was detected for the control GST-column or the GST-CBP I (residues 461 to 662) column. (**Fig. 7c**) Comparison of the HPV-16 E6-CBP II interaction with known E1A-CBP II and HPV-16 E6-E6AP interactions using GST micro-affinity column assays. (**Fig. 7d**) Demonstration of a direct interaction between HPV-16 E6 and CBP using two recombinant bacterially expressed proteins. GST or GST-E6 was passed over a column containing MBP-CBP (residues 1808 - 1852) fusion protein. Bound GST-fusion protein was detected by western analysis using a specific GST antibody. The MBP-CBP fusion protein was also passed over a GST or GST-E6 column and the interaction detected using an MBP antibody.

Figure 8. Identification of an HPV-16 E6 binding site on CBP/p300. Shown is a schematic representation of GST-CBP fusion constructs used in micro-affinity column experiments used to define CBP sequences capable of binding HPV-16 E6. Demonstrated is the ability of a 19 amino acid sequence of CBP (residues 1808-1826) to bind in vitro translated HPV-16 E6 protein (lane 7). Deletion into these sequences

abolishes E6 binding (lane 8). Also shown is an alignment of the identified E6-binding site within CBP and a comparison with the corresponding p300 sequence. An asterix represents the conservation of an identical amino acid residue in that position, while a "+" represents a conservative change. An E1A peptide that can bind this 19 amino acid CBP sequence can inhibit the E6-CBP interaction, while a CBP-binding deficient mutant peptide cannot.

Figures 9a-9c. Mapping of an HPV-16 E6 region involved in the interaction with CBP. (**Fig. 9a**) Amino acid sequence of the HPV-16 E6 protein indicating the two zinc finger structures present in this protein. Indicated are the numbers of the amino acid residues which mark start or end points of HPV-16 E6 fragments used in interaction studies. (**Fig. 9b**) Schematic representation of GST-E6 fusion constructs used in micro-affinity column assays. (**Fig. 9c**) Interaction experiments define a region between HPV-16 E6 residues 100-147 as sufficient for the binding of CBP.

Figures 10a and 10b. The E6-CBP/p300 interaction is specific for "high-risk" HPV E6 proteins. (**Fig. 10a**) Micro-affinity column experiments using either GST-fusion or in vitro translated E6 proteins demonstrate that only E6 proteins from the high-risk HPV types 16 and 18, but not the low-risk HPV types 6 and 11, are capable of interacting with CBP. (**Fig. 10b**) Mammalian two-hybrid experiments (described in Material and

Methods) and shown schematically here, indicate that the distinction between high-risk and low-risk E6 proteins extends to the in vivo interaction with the CBP II domain. Activation of the G5E1BCAT reporter is only seen after co-transfection of GAL4-16 E6 and CBP II-VP16, and not for those experiments in which GAL4-11 E6 or CBP I-VP16 proteins were expressed.

Figures 11a-11c. The HPV-16 E6 mutant L50G binds CBP, but is unable to interact with E6AP or degrade p53 in vitro. (**Fig. 11a**) Schematic representation of the HPV-16 E6 mutant L50G showing the position of the amino acid exchange in the first zinc finger (marked by a +) and the identified CBP-interaction domain within the second zinc finger (bold line). GST micro-affinity column experiments using in vitro translated HPV-16 E6 L50G protein demonstrate the ability of this mutant to interact with GST-CBP. (**Fig. 11b**) Similar in vitro micro-affinity column experiments show that unlike the WT 16 E6 protein, but similar to HPV-11 E6, the HPV-16 E6 mutant L50G is unable to interact with GST-E6AP. (**Fig. 11c**) p53 degradation assays using in vitro translated ^{35}S-labeled p53 mixed with various in vitro translated E6 proteins. The numbered columns indicate the levels of p53 protein after various incubation times (0, 30, 90, and 180 min) at room temperature.

Figures 12a and 12b. HPV-16 E6 targets the ability of CBP to activate p53-dependent transcription. (**Fig. 12a**) U2-OS cells were transfected with the p53-responsive CAT reporter (PG13) or a control vector with mutated p53 binding sites (MG15). Co-transfection of expression-vectors for viral proteins show that HPV proteins able to interact with CBP can down-regulate p53 transactivation to a level comparable with Ad E1A. (**Fig. 12b**) Over-expression of full-length CBP in experiments similar to those described above show that HPV proteins able to interact with CBP, including the HPV-16 L50G mutant, abolish the CBP-dependent superactivation of p53-dependent transcription seen with full-length CBP alone.

Figure 13. Polylinker of pMALP.